Annual Progress Report
to the
Commission on Environmental Hygiene
of the
Armed Forces Epidemiological Board

Sterilization Action of Chlorine and Iodine on Bacteria and Viruses in Water Systems

For the period: 1 July 1963 to 28 February 1964

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A B S T R A C T

1. The Johns Hopkins University
School of Hygiene and Public Health

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A. Effect of Iodine on Bacterial RNA Virus (f_2)

Iodine reacts best with some viruses at pH values between 6 and 8, and at much higher or lower pH than delimited by the range, the inactivation of virus will be materially reduced.

The presence of the iodide ion will greatly reduce the inactivation of some viruses.

B. Probable Mode of Action of Iodine on Bacterial Virus (f_2)

The reaction of iodine with the sulfhydryl groups of the protein coat apparently is not responsible for the complete inactivation of the virus.

Iodination of the tyrosin was effective provided no significant amount of iodide was in the system.

7. Key words: Water Disinfection, Water Supply
Halogenation
Iodination
Decxyribonucleic Acid
Virus, RNA Bacteriophage

Introduction

This progress report is concerned with the continuation study of the mode of action of iodine in the inactivation of bacteria and viruses. The previous finding revealed that the bactericidal action of iodine is rapid, being complete within one minute. The killing action continues until all iodine is consumed. The ability of the bacterial cell to consume oxygen and to incorporate inorganic phosphate into RNA and DNA is blocked immediately by the action of iodine. Nevertheless, iodine was unable to inactivate the transforming DNA, whereas both chlorine and bromine readily destroyed the transforming activity (see Figure 1). Experiments with I^{131} most of the iodine reacting with the bacterial cell suggest that killing is primarily by oxidation of the sulfhydryl groups rather than by substitution or additions to the bacteria components.

The current studies have concentrated on the effect of iodine on bacterial RNA virus (f_2) and on polio virus. Work with the latter has not progressed sufficiently to include findings since equipment had to be assembled and suitable systems devised for conducting study with polio.

Materials and Methods

RNA bacterial virus f_2 strain was kindly supplied by Dr. N.D. Zinder through Dr. T. Fukasawa. Media and methods were the same as described by Loeb and Zinder (1). The f_2 bacterial virus was precipitated with 2M concentration of ammonium sulfate and resuspended in 0.05M phosphate buffer, pH 7, containing 0.14M saline (PBS). The bacterial virus stock solution, containing approximately 10^{13} PFU/ml, was diluted to 10^{-4} and used as a test virus. In some of the later experiments a CsCl purified phage was used as test strain. Residual iodine was detected with Paragon (Eastern Chemical Company) indicator.

In order to terminate iodine reaction, sodium thoisulfate was used for initial experiments. In most experiments, however, thoisulfate was not necessary since mixing test solutions into complexing media instantly reduced the active iodine to iodide. Para-chloromercuribenzoic acid, obtained from the Sigma Company, Inc., was diluted in 0.05M tris-HCl buffer (pH 8) containing 0.14M NaCl. For reaction at different pH levels, acetate, phosphate, tris-HCl, glycine buffer was used in proper range of pH, each containing 0.14M of saline.

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(1)Loeb, T. and Zinder, N. D., A Bacteriophage Containing RNA, PNAS, 47; 282, 1961

Iodotyrosin was measured in increments at 312 millimicrons wave length (2).

Results

The inactivation of both Esch. coli and the bacterial virus was complete at room temperature within 30 seconds with a 0.04 mM dosage of iodine (10 mg/l) in the presence of 0.048 mM of iodide. It was observed, however, that small increases in iodide concentration altered the rate of virus inactivation but did not materially reduce the effect on Esch. coli. Even at 0°C in the presence of 0.5M iodide E. coli was inactivated within five minutes, whereas the bacterial virus was almost fully active during one hour of contact. These results are shown in Figure 2.

Using a constant dosage of iodine of 0.04 mM at room temperature the relationship of varying concentration of iodide and survival of virus is given in Figure 3. After 10 minutes of contact a striking increase in surviving virus fraction is obtained by increasing the concentration of KI from 10^{-3}M to 10^{-2}M. If the concentration of KI is increased above 10^{-2}M, more than 10% of the initial inoculum will survive.

It can be seen from virus survivals obtained at the end of 5, 30 and 60 minutes of contact time that the concentration of KI alters the rate of virus inactivation by iodine, as given on the right graph in Figure 3.

The hydrogen ion concentration exerts a profound influence on the action of chlorine and may be expected to similarly affect the disinfection properties of iodine. The results, however, with virus inactivation were somewhat unexpected. Figure 4 shows the survival fraction of virus at different pH values after constant amounts of virus were reacted with the same iodine dosage of 0.04 mM in the presence of 0.048 mM of iodide. The survival of virus varied directly with the hydrogen ion concentration. Below pH 6 there was an increase in the survival fraction, reaching a maximum at pH 4 to 5 where more than 0.1% of the virus remained active after 10 minutes of contact time.

Para-chloromercuribenzoic acid (PCMB) is a sulfhydryl attacking agent. Esch. coli and virus were treated with a 10^{-3}M concentration of PCMB at room temperature and pH 8.0 for one hour. The survival of

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(2)Cha, C. Y. and Scheraga, H. A., Differentiation of the Tyrosyl Group of Ribonuclease A by Iodination, Bioch. Biophy. Research Commun. 5; 67, 1961

organisms have been plotted in Figure 5. It is apparent that while E. coli was successfully inactivated within a few seconds the virus remained active for more than one hour of contact.

Tyrosin, another protein residue in the coat of the bacterial virus, may be involved in interaction with iodine. Experimental results, also shown in Figure 5, indicate that iodination is complete providing the iodide ion is kept below the 10^{-4}M level. The rate of reaction is greatly decreased as the concentration of KI increased.

A similar observation has been made using polio virus and will be reported by Nomura and Hsu at a later date.

Discussion of Results

In discussing the reaction of iodine with viruses it would be of interest to briefly review the current theory regarding the interaction of iodine with water. At the present time the active iodine species capable of reacting with the complex protein coat is the cationic hydrated iodine, H_2OI^+(3,4). It would not be consistent to believe that non-ionic or anionic forms of iodine would possess the reacting power for the modification of proteins.

Before considering the active species (H_2OI^+) the presence of hydroxyl ions should be mentioned in the formation of HOI, hypoidous acid.

$$I_2 + OH^- \rightleftharpoons HOI + I^- , \qquad K = 30 \qquad (1)$$

$$HOI \rightleftharpoons H^+ + OI^- , \qquad K_2 = 10^{-11} \qquad (2)$$

From these equilibrium equations the ratio of HOI:I_2 can be computed (5). As the hydroxyl ion concentration is increased the HOI is further oxidized as given below:

$$3HOI + 2(OH^-) \rightleftharpoons HIO_3 + 2I^- + 2H_2O \qquad (3)$$

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(3)Bell, R. P. and Gelles, E., The Halogen Cations in Aqueous Solution, J. Chem. Soc., 1951, 2734, (1951)
(4)Hughes, W. L., Chemistry of Iodination, Ann. N.Y. Acad. Sci. p.3, 1957-58
(5)Chang, S. L., The Use of Active Iodine as a Water Disinfectant, J. Am. Pharmaceutical Assoc. 47; 417, 1958

The active species of the hydrated iodine cation, H_2OI^+ is produced by the following reaction (3):

$$I_2 + H_2O \rightleftharpoons H_2OI^+ + I^-, \quad K = 1.2 \times 10^{-11} \quad (4)$$

and reaction (5) shows the combining of I_2 with iodide to form the inactive tri-iodide:

$$I_2 + I^- \rightleftharpoons I_3^-, \quad K_2 = 7.14 \times 10^2 \quad (5)$$

From the equations (4) and (5) the rate of reaction must vary inversely as the square of the concentration of iodide (4):

$$\frac{(H_2OI^+)\ (I^-)^2}{(I_3^-)\ (H_2O)} = K/K_2$$

and by increasing the I^- by even small amounts the rate of iodine reaction will decrease rapidly.

In addition, since iodine is soluble only to the extent of 1.1 mM/liter in water at 20°C iodide is normally added to produce soluble complexes which increases the solubility of iodine. Most of the experiments in this study were performed at pH 7 with 0.04 mM (10 ppm) iodine in the presence of 0.048 mM KI. Under these conditions 90% of the iodine exists as I_2 and free to react with water to give active (H_2OI^+).

The recent report (6) that organic iodine (Wescodyne) was unable to completely inactivate polio virus in the presence of organic substances may be explained by the presence of iodide. The iodide reduced from the iodine by the organic substance will inhibit the viricidal action as observed above.

There is evidence available that the biological activity of TMV-RNA (1) and the transforming DNA is not destroyed by iodine. The inactivation of virus by iodine is conceivably through the modification of the protein coat. The bacterial virus f_2 has no histidine residue in the protein coat (7) and the iodination of the sulfhydryl, tryptophanyl and tyrosyl groups need only be considered with this virus.

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(6) Wallis, C., Bahbahani, A. M., Lee, L. H., and Bianchi, M., The Ineffectiveness of Organic Iodine (Wescodyne) as a Viral Disinfectant, Am. J. Hyg., 78; 325, 1963

(7) Nathans, D., Notani, G., Schwartz, J. H. and Zinder, N. D., Biosynthesis of the Coat Protein of Coliphage f_2 by E. coli extracts PNAS, 48; 1424, 1962

It was evident from experimental results with p-chloromercuribenzoic acid, that the modification of the sulfhydryl groups was insufficient to completely inactivate the f_2 virus. In this case inactivation may be explained by one or more of the following:

1. -SH groups are not essential for absorption and ejection of nucleic acid,

2. -SH groups are buried deep in molecule and are not accessible by iodine, and

3. protein coat does not contain -SH groups.

Choppin, et. al. (8) and Allison (9) have reported that some viruses are resistant to the -SH reacting agent. In contrast, Esch. coli was inactivated completely since the -SH groups in bacteria perform many indispensible enzyme functions.

At pH 7 the rate of iodination of tyrosin was rapid providing the iodide ion in the system was kept below 10^{-4}M concentration. Again this is a phenomenon of maintaining iodine in active cationic form. It may be reasoned that the cationic iodine will readily combine with the phenolate ion of tyrosin, (R -  - O^-) or the quinonoid form (R -  = O) in alkaline water. The iodination of tyrosin has been shown to vary inversely with the hydrogen ion concentration by Li (10) and the rates should be greater as the OH^- concentration of waters increase beyond pH 5.0. It must be assumed that the mode of virus inactivation observed in these experiments was due, in part, to the iodination of the tyrosyl amino acid residue of the protein coat.

Esch. coli was rapidly and completely inactivated by iodine even at 0°C in the presence of high concentrations of iodide. It is likely that the high sensitivity of the sulfhydryl group to even depressed concentrations of H_2OI^+ is sufficient to modify the enzymes located on the surface of the cell, which carry indispensible metabolic functions and, without which, cause the death of the organism.

The conclusions are given as the Abstract at the beginning of this report.

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(8) Choppin, P. W. and Philipson (Rockefeller Inst.) The Inactivation of Enterovirus Infectivity by the Sulfhydryl Reagent p-chloromercuribenzoate, J. Exp. Med., 113; 713-734, 1961: 112; 445, 1960. On the Role of Virus Sulfhydryl Groups in the Attachment of Enterovirus Erythrocytes

(9) Allison, A. C., Observation on the Inactivation of Viruses by Sulfhydryl Reagents, Virology, 17; 176-183, 1962

(10) Li, C. H., J. Am. Chem. Soc., 64; 1147, 1942, Kinetics and Mechanisms of 2.6 - di-iodotyrosine Formation

Summary

A. Effect of Iodine on Bacterial RNA Virus (f_2)

In the practical application of iodine as a viricidal agent two problems must be recognized.

1. Iodine reacts best with some viruses at pH values between 6 and 8, and at much higher or lower pH values than delimited by this range, the inactivation will be materially reduced.

2. The inactivation rate of some viruses by iodine is greatly reduced by the presence of the iodide ion. The phenomenon is most likely due to the suppression of active species of H_2OI^+ by the iodide and the formation of inactive ions such as tri-iodide. Therefore, iodine solutions should not be employed as viricidal agents in the presence of organic substances such as culture media, serum and sewage which have been shown capable of reducing the iodine to iodide in concentrations greater than 10^{-3} to 10^{-2} M and may limit virus inactivation to only 90% of the initial numbers of virus particles.

B. Probable Mode of Action of Iodine on Bacterial Virus(f_2)

Since it has been shown that iodine does not inactivate the biological activity of Tobacco Mosaic Virus - RNA (11) and the transforming DNA, it is conceivable that the inactivation of bacterial virus is due to the modification of the protein coat. For the (f_2) bacterial virus under study only the reaction of iodine with the sulfhydryl, tryptophanyl and tyrosyl groups need be considered.

Reaction of iodine with the sulfhydryl groups apparently is not responsible for the complete inactivation of the virus since the virus was resistant to p-chloromercuribenzoic acid, a -SH group reaction agent.

Iodination of tyrosin with cationic hydrated iodine was effective provided no significant amounts of iodide was in the system. Li (10) has reported that the rate of tyrosin iodination varies inversely with the hydrogen ion concentration. This may account for the poor inactivation of the virus in systems with pH values below 6.0.

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(11) Brammer, K. W., Chemical Modification of Viral Ribonucleic Acid, B.B.A. 72; 217, 1963. Virus Laboratory, Univ. of Calif., Berkeley, Calif.

FIGURE 1

EFFECT OF IODINE, CHLORINE, AND BROMINE ON PURIFIED TRANSFORMING DNA

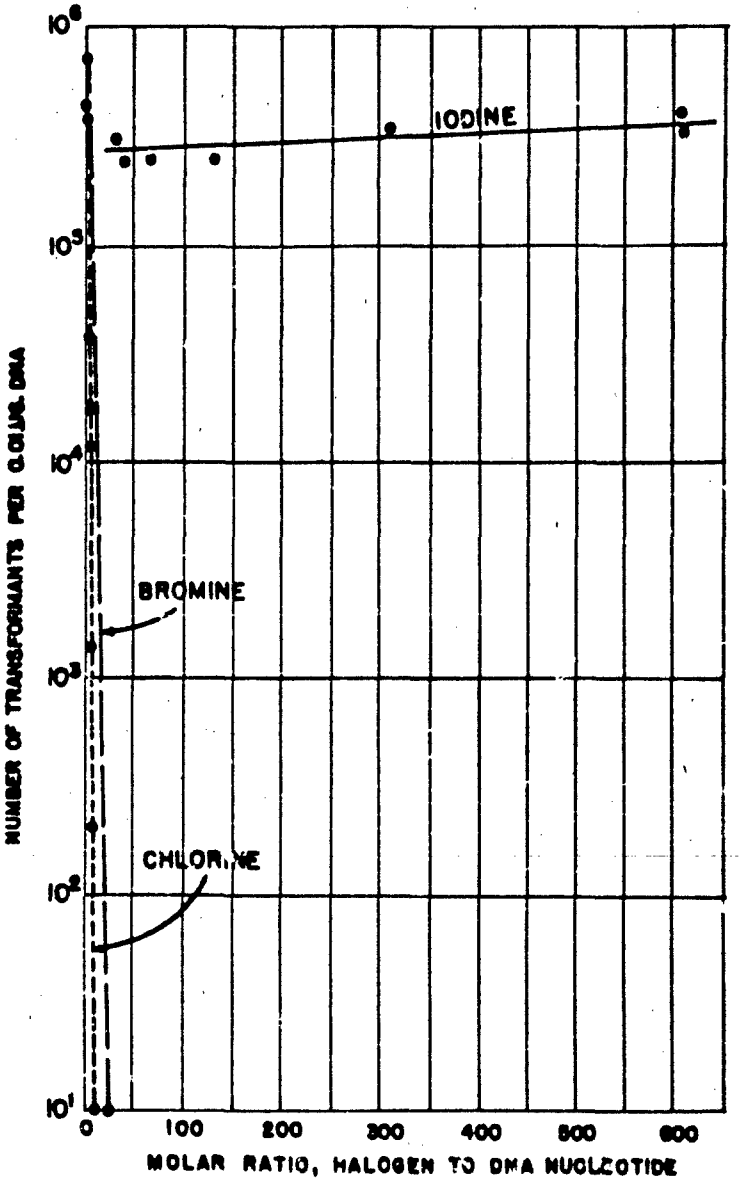
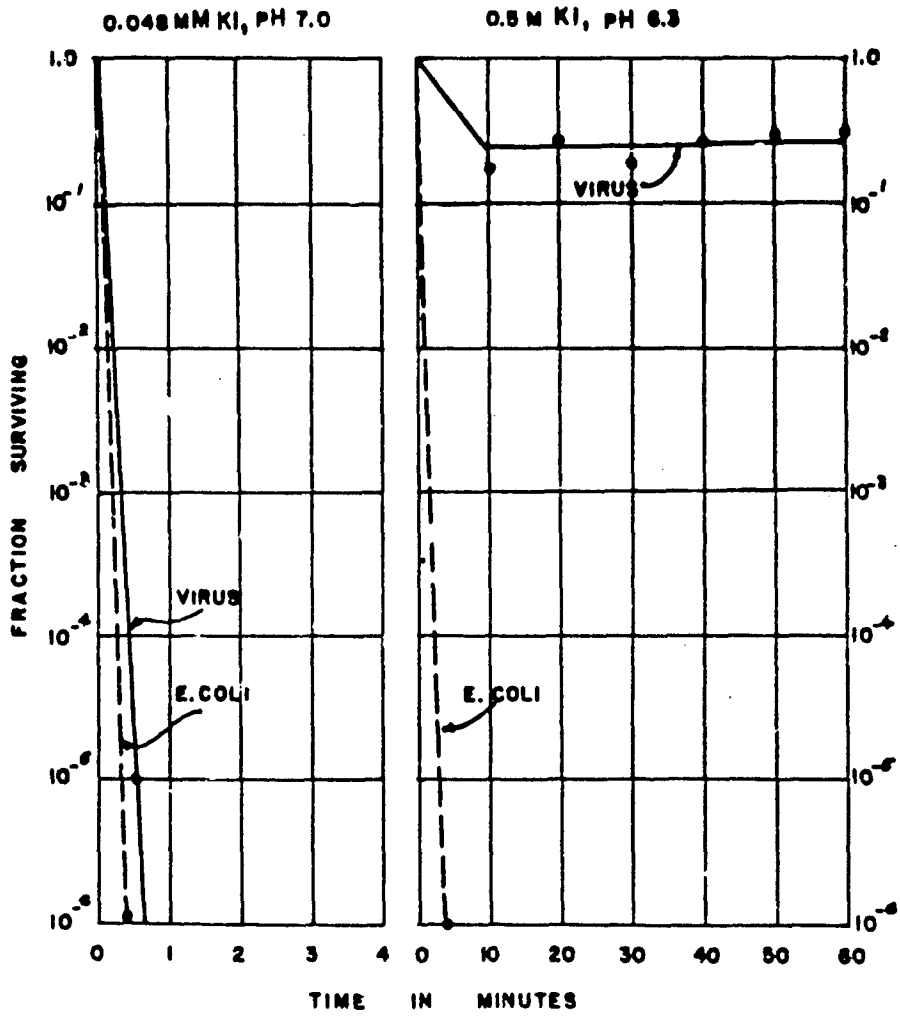
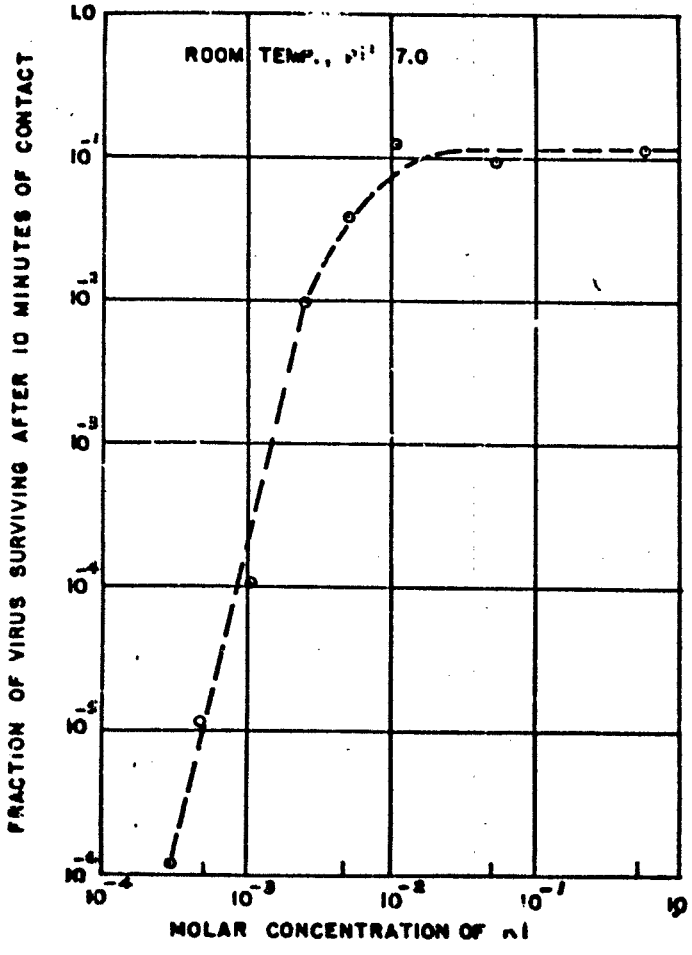


FIGURE 2

SURVIVAL OF BACTERIAL VIRUS AND E.COLI TREATED WITH 0.04 mM OF IODINE (10 MG./L) SHOWING THE ROLE OF I^- ON INACTIVATION



EFFECT OF KI CONCENTRATION ON RATE OF VIRUS INACTIVATION WITH 0.04 MM OF IODINE



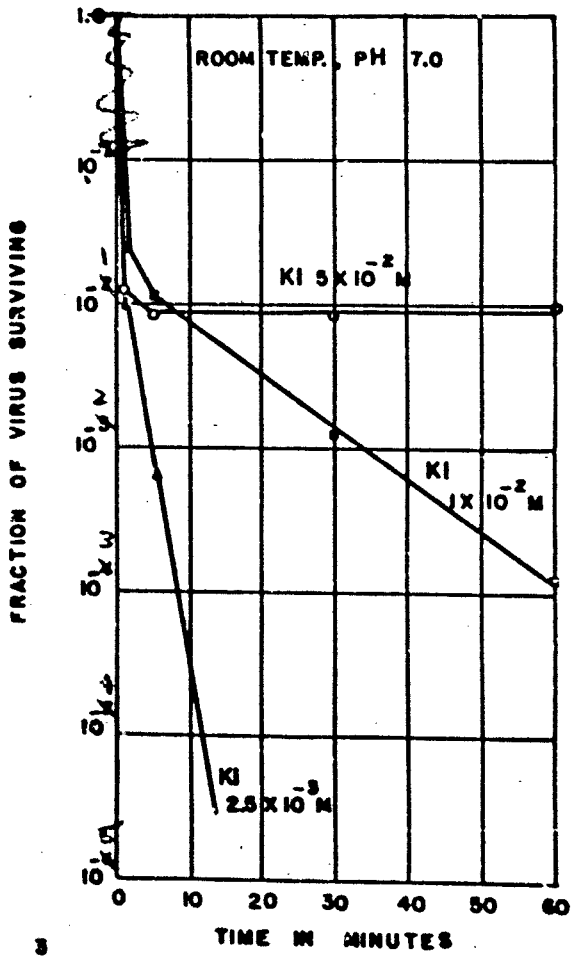


FIGURE 3

FIGURE 4

EFFECT OF pH ON SURVIVAL OF BACTERIAL VIRUS WHEN TREATED WITH 0.04 mM IODINE AND 0.048 mM IODIDE

ROOM TEMPERATURE

INITIAL INOCULUM 5×10^8/ML.

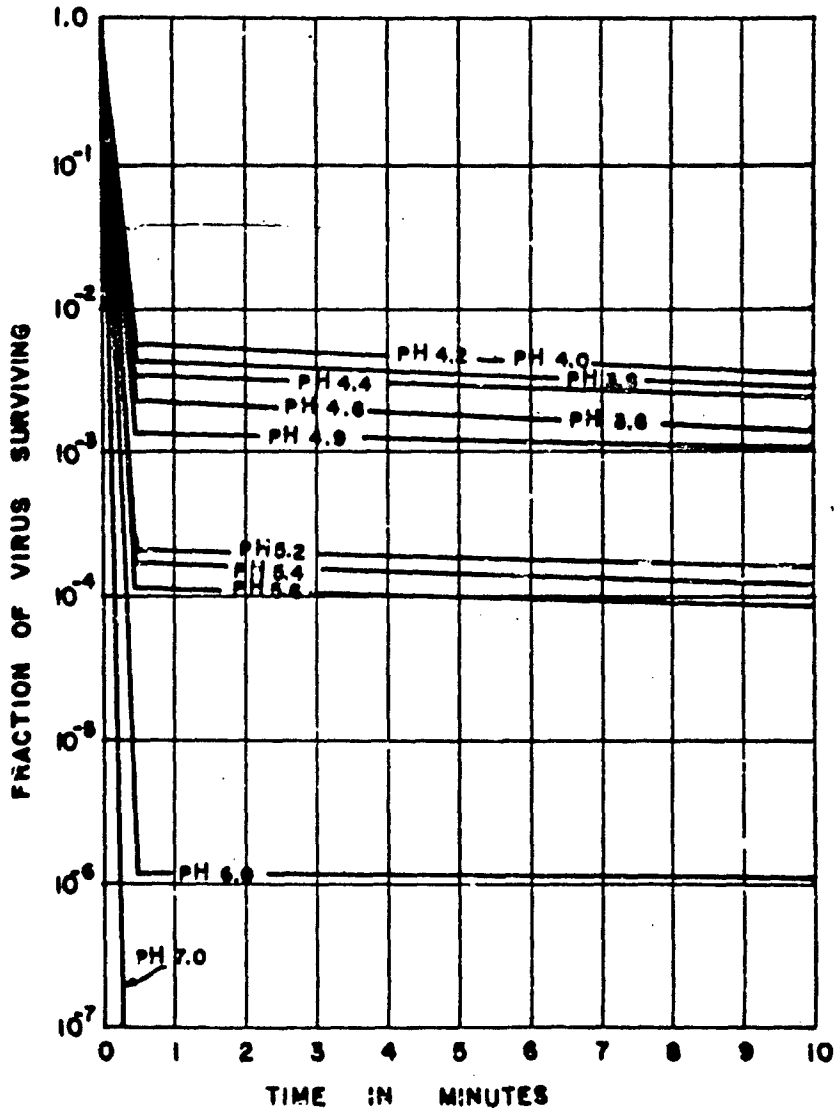
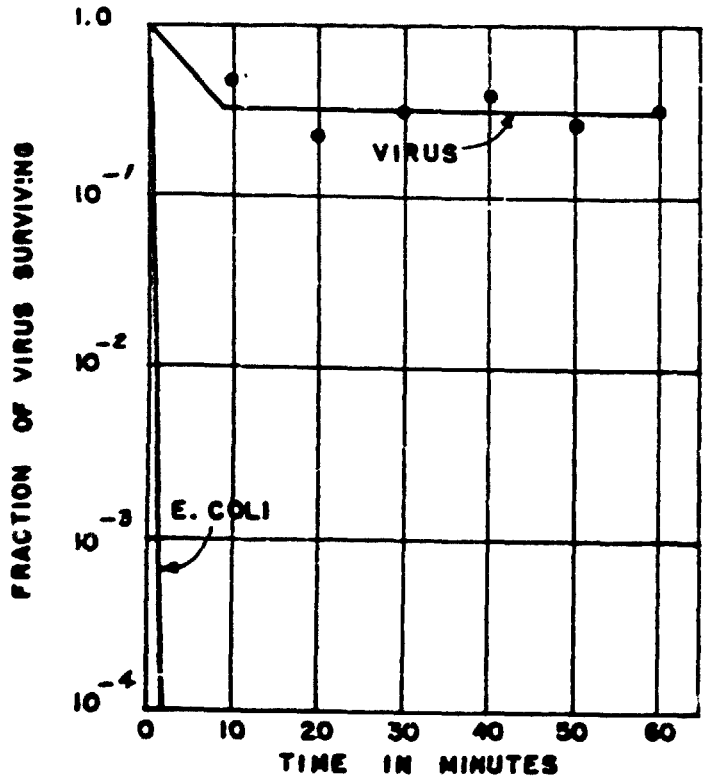


FIGURE 5

EFFECT OF P-CHLOROMERCURIBENZOIC ACID (SULFHYDRYL REACTING AGENT) ON VIRUS AND E. COLI

10^{-3} M PCMB, ROOM TEMP., PH 8.0



EFFECT OF VARYING AMOUNTS OF KI ON THE IODINATION OF TYROSIN

1.25×10^{-4} M L-TYROSIN
5.00×10^{-4} M IODINE

ROOM TEMP., PH 7.0